L2 ANSWER 7 OF 15 BIOSIS COPYRIGHT (c) 2006 The Thomson Corporation on STN
DUPLICATE 4
TI Analysis of chondrex (**YKL-40**, HC gp-39) in the cerebrospinal fluid of patients with spine disease.
AU Tsuji, Taichi [Reprint author]; Matsuyama, Yukihiro; Natsume, Naoki; Hasegawa, Yukiharu; Kondo, Seiji; Kawakami, Hiroshi; Yoshihara, Hisatake; Iwata, Hisashi
SO Spine, (April 1, 2002) Vol. 27, No. 7, pp. 732-735. print.
CODEN: SPINDD. ISSN: 0362-2436.
AB Study Design: The expression of chondrex (**YKL-40**, HC gp-39) was measured in the cerebrospinal fluid of from patients with spine diseases. Objectives: To quantify the levels of chondrex in human cerebrospinal fluid, and to clarify the nature of its expression. Summary of Background Data: Chondrex is a newly discovered 40-kDa glycoprotein identified originally in the whey secretions of nonlactating cows. It is secreted by a human osteosarcoma cell line, human articular cartilage chondrocytes, and human fibroblasts. However, the function of chondrex in chondrogenesis is unknown, and the expression of chondrex in human cerebrospinal fluid has never been reported. Methods: The concentration of chondrex in human cerebrospinal fluid was measured by sandwich immunoassay with antihuman chondrex **antibodies**. Cerebrospinal fluid samples were collected from two groups of patients. Group 1, the control group, consisted of 34 trauma patients. Group 2 consisted of 130 patients with spine diseases: 29 with cervical spondylotic myelopathy, 30 with lumbar disc herniation, 35 with lumbar canal stenosis, and 36 with scoliosis. All values are expressed as the mean+-standard deviation. Results: The concentration of chondrex in Group 1 (control group) was 113.8+-48.3 ng/mL. The concentrations of chondrex in Group 2 were 245.3+-107.2 ng/mL in cervical myelopathy, 143.2+-53.6 ng/mL in lumbar disc herniation, 241.5+-77.2 ng/mL in lumbar canal stenosis, and 71.4+-33.9 ng/mL in scoliosis. The concentrations of chondrex in cervical myelopathy, lumbar canal stenosis, and lumbar disc herniation were significantly higher than in the control group ($P<0.05$). Conclusions: In this study, the chondrex concentration was high in spine diseases causing spinal stenosis. The authors believe that chondrex is expressed in cerebrospinal fluid as a result of damage or stress to the neural structure, and that it could be a new marker for spine diseases.

L2 ANSWER 8 OF 15 CAPLUS COPYRIGHT 2006 ACS on STN
TI Serum **YKL-40** associated with osteoarthritis and atherosclerosis in nonhuman primates
AU Register, Thomas C.; Carlson, Cathy S.; Adams, Michael R.
SO Clinical Chemistry (Washington, DC, United States) (2001), 47(12), 2159-2161
CODEN: CLCHAU; ISSN: 0009-9147
AB The assocns. of **YKL-40** in serum and synovial fluid with naturally occurring osteoarthritis (OA) and diet-induced atherosclerosis in cynomolgus monkeys were determined Before necropsy, the hips, knees, and feet of the animals were radiographed and sera collected and stored at -70°. Synovial fluid was obtained from the knee joint by injection of 3 mL of sterile saline solution into the joint followed by aspiration, after which the knee joints were collected. **YKL-40** was measured in coded samples by a sandwich ELISA with a monoclonal anti-**YKL-40** capture **antibody** for detection, and p-nitrophenylphosphate as substrate. **YKL-40** was measured in 13 serum and 16 synovial fluid samples from female cynomolgus macaques. Serum and synovial fluid of **YKL-**

40 were highly correlated. Among animals with no detectable OA of the knee, the extent of coronary artery atherosclerosis was significantly correlated with serum YKL-40. YKL-40 appears to be a serum and synovial fluid marker of vascular and other diseases in which inflammation and/or tissue remodeling occur.

L2 ANSWER 10 OF 15 BIOSIS COPYRIGHT (c) 2006 The Thomson Corporation on STN DUPLICATE 6

TI Concentration and localization of **YKL-40** in hip joint diseases.

AU Kawasaki, Masashi [Reprint author]; Hasegawa, Yukiharu; Kondo, Seiji; Iwata, Hisashi

SO Journal of Rheumatology, (February, 2001) Vol. 28, No. 2, pp. 341-345. print.
CODEN: JRHUA9. ISSN: 0315-162X.

AB Objective. **YKL-40** is a major secretory protein from human chondrocytes and synovial fibroblasts. We evaluated the concentrations and localization of **YKL-40** in hip joint diseases, and analyzed the possibility of **YKL-40** as a new inflammatory joint marker. Methods. The concentration of **YKL-40** in synovial fluid (SF) was measured by a sandwich-type ELISA. SF samples were collected from 19 hips with osteoarthritis (OA) of the hip joint, 21 hips with osteonecrosis of the femoral head (ONFH), and 5 hips with failed total hip arthroplasty (failed THA). In all cases of failed THA, cartilage tissue in hip joints was removed completely during the previous THA. The localization of **YKL-40** was determined through immunohistochemical analysis using a specific **antibody**. Results. The mean SF concentration of **YKL-40** was significantly higher in ONFH and failed THA than in OA. Comparison by OA grade was not significantly different. In staging of ONFH, Ficat stage III with collapsed femoral head showed significantly higher **YKL-40** concentrations than the other stages. Immunohistochemical studies showed that **YKL-40** was localized in chondrocytes in the superficial and middle layers of the cartilage. In the synovium, **YKL-40** was localized in fibroblasts and macrophages. Conclusion. **YKL-40** reflects the degree of inflammation rather than cartilage metabolism. **YKL-40** may be a useful inflammatory marker of hip joint diseases.

L2 ANSWER 11 OF 15 BIOSIS COPYRIGHT (c) 2006 The Thomson Corporation on STN DUPLICATE 7

TI Development of an enzyme-linked immunoassay for the quantification of **YKL-40** (cartilage gp-39) in guinea pig serum using hen egg yolk **antibodies**.

AU De Ceuninck, Frederic [Reprint author]; Pastoureau, Philippe; Agnellet, Severine; Bonnet, Jacqueline; Vanhoutte, Paul Michel

SO Journal of Immunological Methods, (1 June, 2001) Vol. 252, No. 1-2, pp. 153-161. print.
CODEN: JIMMBG. ISSN: 0022-1759.

AB An indirect competition immunoassay for the quantification of **YKL-40** (cartilage gp-39, Chondrex) in guinea pig serum has been developed using egg yolk **antibodies** (IgY). The immune response of hens to **YKL-40** was verified by immunoblot analyses. Highly specific **antibodies** were obtained 30 days after the first injection. The ELISA was developed in 96-well microtiter plates with quadruplicate determinations for each point. The assay was based on the ability of **YKL-40** present in serum to displace the binding of **antibodies** to the coated antigen. An inhibition mixture containing standard **YKL-40** or guinea pig serum, diluted 1/5, and primary **antibodies**, diluted 1/5000, was allowed to equilibrate for 2 h at room temperature and dispensed for 16 h at 4degreeC in wells coated with 1 mug/ml of **YKL-40**.

Detection was achieved by the addition of rabbit anti-chicken **antibodies** conjugated to peroxidase followed by tetramethylbenzidine. Specificity was assessed by parallelism between a dilution curve of serum and standard **YKL-40**. The sensitivity of detection was 10 ng/ml. Intra- and interassay coefficients of variation were both 8.7%. The analytical recovery was 101.5 +- 5.4% (mean +- standard deviation (SD), n = 9). The **YKL-40** concentration in serum from 12 adult guinea pigs was 330 +- 216 ng/ml (mean +- SD) with a lower value of 164 ng/ml and an upper value of 982 ng/ml. In contrast to the rat, a dilution curve of rabbit serum gave parallelism with the guinea pig standard, suggesting recognition of a similar epitope. Possible applications of the assay in the guinea pig include disease models where **YKL-40** is overexpressed and could be used as a marker, i.e. osteoarthritis, rheumatoid arthritis, cancer, liver fibrosis, atherosclerosis and more generally, pathologies with increased tissue remodeling.

L2 ANSWER 12 OF 15 BIOSIS COPYRIGHT (c) 2006 The Thomson Corporation on STN DUPLICATE 8

TI Recognition of YKL-39, a human cartilage related protein, as a target antigen in patients with rheumatoid arthritis.

AU Sekine, T.; Masuko-Hongo, K.; Matsui, T.; Asahara, H.; Takigawa, M.; Nishioka, K.; Kato, T. [Reprint author]

SO Annals of the Rheumatic Diseases, (January, 2001) Vol. 60, No. 1, pp. 49-54. print.
CODEN: ARDIAO. ISSN: 0003-4967.

AB Objective: To investigate whether autoimmunity to YKL-39, a recently cloned cartilage protein, occurs in patients with rheumatoid arthritis (RA). Methods: Autoantibody to YKL-39 was assayed by enzyme linked immunosorbent assay (ELISA) and western blotting in serum samples from patients with RA, systemic lupus erythematosus (SLE), and healthy donors, using recombinant YKL-39 protein. This reactivity was compared with that against a YKL-39 homologue, **YKL-40** (human cartilage gp-39/chondrex), which has been reported to be an autoantigen in RA. Results: Autoantibody to YKL-39 was detected in seven of 87 patients with RA (8%), but not in serum samples from patients with SLE or healthy donors. **YKL-40** reactivity was found in only one of 87 RA serum samples (1%), with no cross reactivity to YKL-39. Conclusion: The existence of anti-YKL-39 **antibody** in a subset of patients with RA is reported here for the first time. Further, it was shown that the immune response to YKL-39 was independent of that to **YKL-40**. Clarification of the **antibody** and T cell responses to autoantigens derived from chondrocyte, cartilage, or other joint components may lead to a better understanding of the pathophysiology of joint destruction in patients with RA.

L2 ANSWER 13 OF 15 CAPLUS COPYRIGHT 2006 ACS on STN DUPLICATE 9

TI **YKL-40** as a marker and prognostic indicator for cancers

IN Price, Paul A.; Johansen, Julia S.

SO PCT Int. Appl., 111 pp.
CODEN: PIXXD2

AB This invention provides methods for detecting cancers and for evaluating the prognosis of cancer patients. In particular, the methods of this invention utilize **YKL-40** as a marker for the presence or absence of a cancer and for the prognosis (e.g. likelihood of recurrence) of a cancer. Elevated levels of **YKL-40** are indicative of the presence of a cancer in undiagnosed subjects and

indicate likely recurrence of the cancer in subjects diagnosed as having a cancer.

L2 ANSWER 14 OF 15 CAPLUS COPYRIGHT 2006 ACS on STN DUPLICATE 10
TI Assay for protein **YKL-40** as a marker for degradation of mammalian connective tissue matrixes
IN Price, Paul A.; Johansen, Julia S.
SO PCT Int. Appl., 52 pp.
CODEN: PIXXD2
AB A competitive immunoassay is provided for diagnosing a disease state in a mammal associated with degradation of connective tissue in the mammal which contains protein **YKL-40**. The assay can be used e.g. to identify the presence of inflammatory or degenerative joint disease and tumor metastasis (to the extent it can be correlated to serum **YKL-40** levels). Serum **YKL-40** levels are also suggestive of the prognosis for the length of survival in breast cancer patients following recurrence and/or metastasis of their cancers. Thus, protein **YKL-40** was isolated and purified from human osteosarcoma cell line MG63 by heparin affinity chromatog. and radiolabeled with 125I or used to raise **antibodies** in rabbits for the immunoassay.

L2 ANSWER 15 OF 15 CAPLUS COPYRIGHT 2006 ACS on STN DUPLICATE 11
TI Assay for **YKL-40** as a marker for degradation of mammalian connective tissue matrixes
IN Price, Paul A.; Johansen, Julia S.
SO PCT Int. Appl., 88 pp.
CODEN: PIXXD2
AB The invention is an immunoassay, and especially a RIA, for identifying the presence of, and monitoring, a disease state in a mammal which is associated with degradation of connective tissue in the mammal. The method uses an **antibody** specific for **YKL-40** to detect and determine whether diagnostically or prognostically significant levels of **YKL-40** protein and/or **YKL-40** peptide are present in a biol. sample. The method can be used, for example, to identify the presence of inflammatory or degenerative joint disease or degeneration of connective tissue in organs. Serum **YKL-40** levels as detected and quantified by the inventive method are also suggestive of the prognosis for the length of survival in breast cancer patients following recurrence and/or metastasis of their cancers. Substantially pure serum **YKL-40** was shown by gel filtration.

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Johansen *et al. Plasma YKL-40 concentrations in patients with rheumatoid arthritis*, Abstract for scientific Conference published on or after July 12, 1992 in Davos, Switzerland.

PLASMA YKL-40 CONC. IN PATIENTS WITH RHEUMATOID ARTHRITIS. CHANGES DURING PULSE TREATMENT WITH METHYLPREDNISOLONE.

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YKL-40 is a glycoprotein that has recently been isolated from the conditioned medium of normal human synovial cells (Nyirkos P et al. Biochem J 1990;268:265) and chondrocytes (personal observation). YKL-40 has not been detected in conditioned medium from human skin and lung fibroblasts. We have termed this new protein YKL-40 based on the one letter code for its first three N terminal aminoacids and its apparent molecular weight. The function and tissue distribution of YKL-40 is at present unknown. Only the first 23 N terminal residues of human YKL-40 has been sequenced. We have developed a specific radioimmunoassay for human YKL-40 and have detected YKL-40 in blood and synovial fluid. We describe here our first studies of plasma YKL-40 levels in patients with rheumatoid arthritis (RA).

Plasma YKL-40 was measured in 97 patients with active RA. The patients entered a double blind placebo controlled trial of pulse treatment with 1000 mg i.v. methylprednisolone (MP) every 4 weeks for six months, followed by 6 months without MP therapy (Hansen TM et al. Br Med J 1990;301:268). The initial level of plasma YKL-40 was 174 ug/L (108-261 ug/L) (median (95% confidence limit)) in the patients with RA and significantly higher ($p<0.001$) than in healthy adults (50 ug/L (36-64 ug/L)). 57 patients completed the trial, taking the same disease modifying drug throughout (31 was treated with MP and 26 with placebo). In the MP treated group a significant decrease ($p<0.01$) was found in YKL-40 24 hours after start of treatment. Furthermore, plasma YKL-40 measured after 4, 8, 12, 16, 20 and 24 weeks of treatment with MP was significantly lower ($p<0.01$ - $p<0.001$) compared to the initial values. 6 months after withdrawal of MP therapy plasma YKL-40 had returned to baseline values. Plasma YKL-40 was unchanged in the placebo group throughout the 12 months study period. The initial plasma YKL-40 conc. in the patients with RA showed a significant correlation with serum CRP ($r=0.52, p<0.001$), serum ESR ($r=0.44, p<0.001$), serum aminoterminal propeptide of type III procollagen ($r=0.47, p<0.001$), serum hyaluronan ($r=0.41, p<0.001$) and with the number of swollen joints ($r=0.37, p<0.001$). At the end of the 12 months study plasma YKL-40 showed a less pronounced correlation with these laboratory parameters.

In conclusion we find that plasma YKL-40 is elevated in patients with active RA and correlated with other parameters of disease activity. MP pulse therapy induced a significant but transient decrease in plasma YKL-40. Like other circulating connective tissue markers (Hørslev-Petersen K et al. Ann Rheum Dis 1988;47:116) plasma YKL-40 levels might be a biochemical marker of connective tissue injury and repair in patients with inflammatory or degenerative rheumatic diseases.

British Journal of Rheumatology 1993;32:949–955

ORIGINAL PAPERS

A NEW BIOCHEMICAL MARKER FOR JOINT INJURY. ANALYSIS OF YKL-40 IN SERUM AND SYNOVIAL FLUID

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SUMMARY

We report the development of the first radioimmunoassay for YKL-40, a M_r = 40 kDa protein which is secreted at high levels by human synovial cells and articular cartilage chondrocytes, and by the human osteosarcoma cell line MG63. This assay uses YKL-40 purified from the conditioned medium of MG63 cells as standard and tracer, and as antigen for immunizing rabbits. With this assay we have discovered high levels of YKL-40 antigen in serum and SF. The molecular weight of serum and SF YKL-40 is identical to purified YKL-40. To evaluate the possible utility of YKL-40 in the assessment of joint disease, we measured YKL-40 in serum and SF of 49 patients with various forms of inflammatory and degenerative joint disease and in the serum of 50 normal adults. The YKL-40 level in serum was significantly higher ($P<0.001$) in the patients compared to the normal adults, but there was no difference in serum YKL-40 between the patients with inflammatory joint diseases and OA. The SF levels of YKL-40 were 15-fold higher than serum levels and there was a significant correlation ($r = 0.55$, $P<0.001$) between YKL-40 concentration in SF and serum. Although the tissue distribution of YKL-40 secretion is presently unknown, these observations suggest that a major portion of serum YKL-40 in fact arises from the joint. Serum and SF YKL-40 levels correlated significantly ($P<0.05$–$P<0.001$) with other indices of joint disease: serum CRP, SF IL-6, and the elastolytic activity of monocytes/macrophages in SF. Serum YKL-40 also correlated with serum PIIINP and elastolytic activity of blood monocytes/macrophages.

These studies indicate that serum and SF YKL-40 levels reflect joint disease and a YKL-40 determination may therefore be useful in the evaluation of connective tissue injury and repair in patients with inflammatory or degenerative rheumatic diseases. Future studies will be needed in order to assess the physiologic significance of elevated YKL-40 levels in patients with rheumatoid diseases.

KEY WORDS: Arthritis; Biochemical markers; Synovial fluid.

YKL-40 is a secreted protein which has recently been isolated from the conditioned medium of normal human synovial cells [1], the human osteosarcoma cell line MG63 [2] and normal human articular chondrocytes (P. A. Price *et al.* in preparation). We have termed this new human protein YKL-40 based on the one letter code for its first three N-terminal amino acids and its apparent molecular weight (40 kDa) [3]. The function and tissue distribution of YKL-40 is at present unknown and only the first 23 N-terminal residues of human YKL-40 have been identified [1, 2].

The evaluation of disease activity and tissue injury in patients with rheumatic diseases requires reliable biochemical markers of inflammation and cartilage destruction. In order to investigate the possible value of YKL-40 in these respects, we have developed a radioimmunoassay (RIA) for analysis of human YKL-40. In the present study we describe the purification of human YKL-40 from the human osteosarcoma cell line MG63, the characteristics of the YKL-40 RIA and the discovery of YKL-40 antigen in serum and SF. To evaluate the utility of YKL-40 in the assessment of joint disease, the levels of YKL-40 were measured in the serum and SF of patients with inflammatory or degenerative joint diseases and in the serum of normal adults. These YKL-40 levels in patients were also correlated with laboratory and clinical measures of inflammation and joint metabolism.

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PATIENTS AND METHODS

Patients

The study comprised 49 patients with inflammatory or degenerative joint diseases. Thirty-four women and 15 men, aged 23–80 yr (median 65 yr). Twenty-nine patients had RA, seven had OA, four had crystal arthritis, two had psoriatic arthritis, five had reactive arthritis and two had monoarthritis. Diagnosis were based on the ARA criteria [4], clinical and radiographic examinations of the knees and direct microscopy of SF. The patients had a serum CRP level of 25–1600 mg/l (median 165). Thirty-four patients were taking NSAIDs and 17 were receiving slow acting anti-rheumatic agents. Fifteen patients had received glucocorticoid therapy systemically or locally within the past 3 months [5]. The inflammation of the knee was evaluated by a clinical index rating from 0–6, consisting of palpable synovial swelling (range 0–3) and pain on palpation (0–3). Informed consent was obtained from all patients in accordance with the Helsinki Declaration II. The study was approved by the regional committee for medical ethics.

0263-7103/93/011949 + 07 $08.00/0

Normal subjects

Control sera were obtained from 50 blood donors, 29 women and 21 men, aged 32–67 yr, who attended the Regional Blood Transfusion Services at Hvidovre Hospital, Denmark. They all were healthy and had normal kidney and liver function. None were taking any medicine.

Collection of serum and SF

Blood samples were allowed to clot at room temperature and then centrifuged at 1500 *g* for 10 min. Knee joint aspirations were performed using conventional aseptic technique without local anaesthesia. The SF was withdrawn as completely as possible using a 1.2-mm gauge needle, and collected in sterile tubes containing ethylenediaminetetraacetate (EDTA, 5 mM final concentration). The SF samples were centrifuged within 1 h of collection at 800 *g* for 20 min for all studies, the supernatant was again centrifuged at 1800 *g* for 30 min in order to remove ex. debris. The samples were either analysed immediately or stored at −80°C for later analysis.

Purification of YKL-40

YKL-40 was purified from serum-free conditioned medium of the human osteosarcoma cell line MG63 (MG63 cells were obtained from the American Type Culture Collection, Rockville, MD). Cells were cultured in 100-mm dishes with RPMI 1640 medium containing 10% newborn calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml vitamin C, and 1 μg/ml vitamin K_1. Cultures were incubated at 37°C in a humidified atmosphere of 10% CO_2. When the cells reached confluence, the culture medium was removed and the cell layer was washed twice with 10 ml of phosphate buffered saline. Ten ml of serum-free RPMI 1640 media containing 50 μg/ml vitamin C and 1 μg/ml vitamin K_1 was then added to each dish. 48 h later, conditioned medium was decanted from each dish and replaced with 10 ml of fresh serum-free medium containing the same level of added constituents. This procedure was repeated every 48 h for up to 10 days. Conditioned medium was freed of cells and debris by centrifugation and stored at −20°C until use. YKL-40 was purified by a modification of the heparin-affinity chromatography method described previously [1]. YKL-40 was first concentrated from 4.75 l of conditioned medium by adsorption to 40 ml (packed volume) of Heparin-Sepharose CL-6B resin by stirring for 2 h at room temperature. The resin was then placed into a 2 × 24 cm column and washed with 3 column vol of 0.01 M sodium phosphate buffer pH 7.4 containing 0.05 M NaCl. YKL-40 was eluted from the resin at room temperature by a linear gradient from 0.05 to 1.2 M NaCl in 0.01 M sodium phosphate buffer pH 7.4 (200 ml each condition). To characterize the purity of YKL-40, 5 μl from every third fraction of the peak fractions from the Heparin-Sepharose CL-6B affinity chromatography were combined with 25 μl SDS loading buffer, electrophoresed on a 5–20% SDS-polyacrylamide gradient gel (BioRad, Laboratories, Richmond, CA), and stained with Coomassie brilliant blue. The concentration of the final YKL-40 used for standards and tracer is based on an absorbance of 1.44 for a 1 mg/ml solution of YKL-40.

Radioimmunoassay of YKL-40

Preparation of radioiodinated YKL-40. Purified YKL-40 was labelled with ^{125}I (sodium salt, Amersham, UK) according to the Iodogen method [6]. Ten μg YKL-40 was incubated for 10 min with 18.5 MBq ^{125}I using 2 μg of iodogen (Pierce and Warriner, Chester Ltd, UK) as oxidant in a reaction volume of 110 μl. Iodination was terminated by moving the reaction mixture from the iodogen tube. The labelled YKL-40 was separated from free iodine by gel filtration using a Sephadex G-25 column (1 × 12.5 cm) equilibrated with assay buffer [16 mM sodium phosphate buffer pH 7.4, 0.12 M NaCl, 0.1% (w/v) human serum albumin]. The calculated specific activity of the labelled antigen was about 15 Ci/g.

Preparation of antibodies. New Zealand white rabbits were immunized by monthly multiple site s.c. or i.m. injection of purified YKL-40. Each injection was made with 0.5 mg of human YKL-40 emulsified in incomplete Freund's adjuvant (1:1). The first four injections were given at intervals of 2 wk and rabbits were bled 10–12 days after the fourth injection. Injections were thereafter given at 4-wk intervals and the animals were bled 10–12 days after each injection.

YKL-40 RIA. YKL-40 antibodies, standards and tracer were diluted in assay buffer. In the assay 100 μl of standards or samples were incubated with 100 μl of YKL-40 antiserum (1:10 000) and 100 μl of YKL-40 tracer (about 15 000 CPM) in a final volume of 400 μl at room temperature for 20–24 h. The antibody-bound tracer was then separated by incubation with 100 μl of Sac-Cel (donkey anti-rabbit antibody coated cellulose suspension; Wellcome Diagnostics Ltd, UK) at room temperature for 30 min. After addition of 1 ml of distilled water the tubes were centrifuged at 2000 *g* for 10 min, the supernatant decanted, and the radioactivity of the precipitate counted in an automatic gamma counter (LKB Wallac, CliniGamma 1272) for the time of 10 000 counts. All standards and samples were assayed in duplicate. The standard curve was constructed by use of a spline function. The precision (intra-assay variation) was calculated from replicate determinations (20 times) on each of three quality control sera in a single assay. The reproducibility (inter-assay variation) was calculated from data obtained during a 5-month period (20 assays) on each of three quality control sera. YKL-40 concentrations in corresponding serum and EDTA plasma samples were compared in 75 blood donors. To assess the effect of freezing and thawing, a fresh serum sample was obtained from six adults and 10 aliquots of each sample were prepared. One aliquot was kept on ice, and the others were frozen at −20°C. At 60-min intervals, the aliquots were removed and thawed at room temperature. One sample was kept on ice and the rest refrozen. This procedure was repeated nine times. To assess the effect of long term storage at

room temperature, a fresh serum sample was obtained from 12 adults, and four aliquots of each sample were prepared. One aliquot was immediately frozen at -20°C, the others were frozen after 24, 48 and 120 h storage at room temperature.

Gel chromatography of serum and SF

The apparent molecular weight of YKL-40 in serum and SF was investigated by comparing the Sephacryl S-300 (Pharmacia, Uppsala, Sweden) elution position of human serum and SF YKL-40 as determined by RIA with the elution position of purified YKL-40. Two ml serum or 1 ml SF from patients with RA were applied on a Sephacryl S-300 column (1.6 × 92 cm; flow rate 14 ml/h; fraction volume 1.8 ml) equilibrated with 5mM NH_4HCO_3.

Other biochemical analyses

Serum CRP was determined by nephelometry (Behringwerke, Marburg, Germany). Interleukin-6 (IL-6) activity was determined by bioassay using the highly specific IL-6 dependent mouse hybridoma cell line B13.29 clone B9 [7]. The aminoterminal propeptide of type III procollagen (PIIINP) was measured by a commercially available RIA (PIIINP RIA-kit, Farmos Diagnostica, Oulunsalo, Finland) [8]. The elastolytic activity of monocytes/macrophages (Mø) were investigated with an assay for live Mø elastolysis [9].

Statistics

Results are expressed as median, range and upper/lower quartile. The statistical significance of the difference between median values was assessed by the Mann-Whitney test. For these analysis values of $P<0.05$ (two-tailed test) were considered significant. The correlations between the different parameters were calculated using the Spearman's rank correlation coefficient. Values are given as Spearman ρ/P value (two-tailed test).

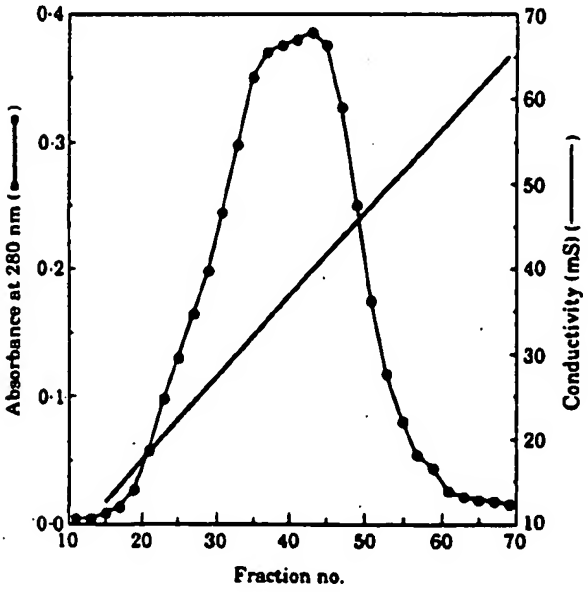

FIG. 1.—Purification of YKL-40 by heparin-affinity chromatography. The YKL-40 in 4.75 l of serum free conditioned medium from MG63 cells was first bound to heparin-Sepharose CL-6B and then eluted by a linear gradient from 0.05 to 1.2 M NaCl in 0.01 M sodium phosphate pH 7.4 (see Methods). Fraction size, 5 ml.

RESULTS

Purification of human YKL-40

YKL-40 was purified from the conditioned medium of MG63 cells since previous studies have shown that YKL-40 is the major protein secreted by these cells [2] and there is as yet no procedure for the isolation of YKL-40 from human tissues. As can be seen in Fig. 1, a single major protein peak was obtained by heparin-affinity chromatography [1] of 4.75 l of serum-free conditioned medium from MG63 cells. SDS-PAGE of the peak fractions revealed the presence of a single protein of M_r = 40 kDa (Fig. 2), and N-terminal protein sequencing of the purified protein yielded a single amino acid sequence (YKLVCYYTSWSQY-REGDGSXFPD). This sequence is identical to the previously published sequence of human YKL-40 [1, 2].

Characteristics of the YKL-40 RIA

Figure 3 demonstrates a typical standard curve for the human YKL-40 RIA. Dose–dilution curves of serum and SF samples from patients with RA and serum samples from healthy adults react indistinguishably from the standard human YKL-40 in the assay. The YKL-40 RIA detects one major peak of antigenicity in the gel filtration of human serum (Fig. 4) and SF (data not shown), and its elution position exactly corresponds to that of purified YKL-40. Crossed immunoelectrophoresis demonstrated that the antibody used in the RIA was monospecific (data not shown).

The sensitivity (detection limit) of this assay, defined as the detectable mass equivalent to twice the standard deviation of the zero binding values, was 10 µg/l. The standard curve was linear between 20 and 100 µg/l. The intra- and interassay coefficients of variance were <6.5 and <12%, respectively. Various amounts of YKL-40 standard (5 ng to 60 ng per tube) were added to four different sera to determine the recovery. The mean analytical recovery was 100.3% (S.D. = 3.0%, n = 16). The YKL-40 level in corresponding serum and EDTA plasma samples was significantly correlated (r = 0.98, $P<0.001$), and YKL-40 was significantly lower ($P<0.001$) in EDTA plasma compared to the corresponding serum value (EDTA plasma YKL-40 = -2.94 + 0.90 x serum YKL-40). In order to test the stability of YKL-40 in blood, serum samples were assayed after repeated freeze–thaw cycles and after long-term storage at room temperature. Through nine freeze–thaw cycles no loss of serum YKL-40 reactivity was found and the YKL-40 reactivity was stable in serum samples stored up to 120 h at room temperature.

Serum concentrations of YKL-40 in patients with joint disease

The individual serum YKL-40 concentrations in the

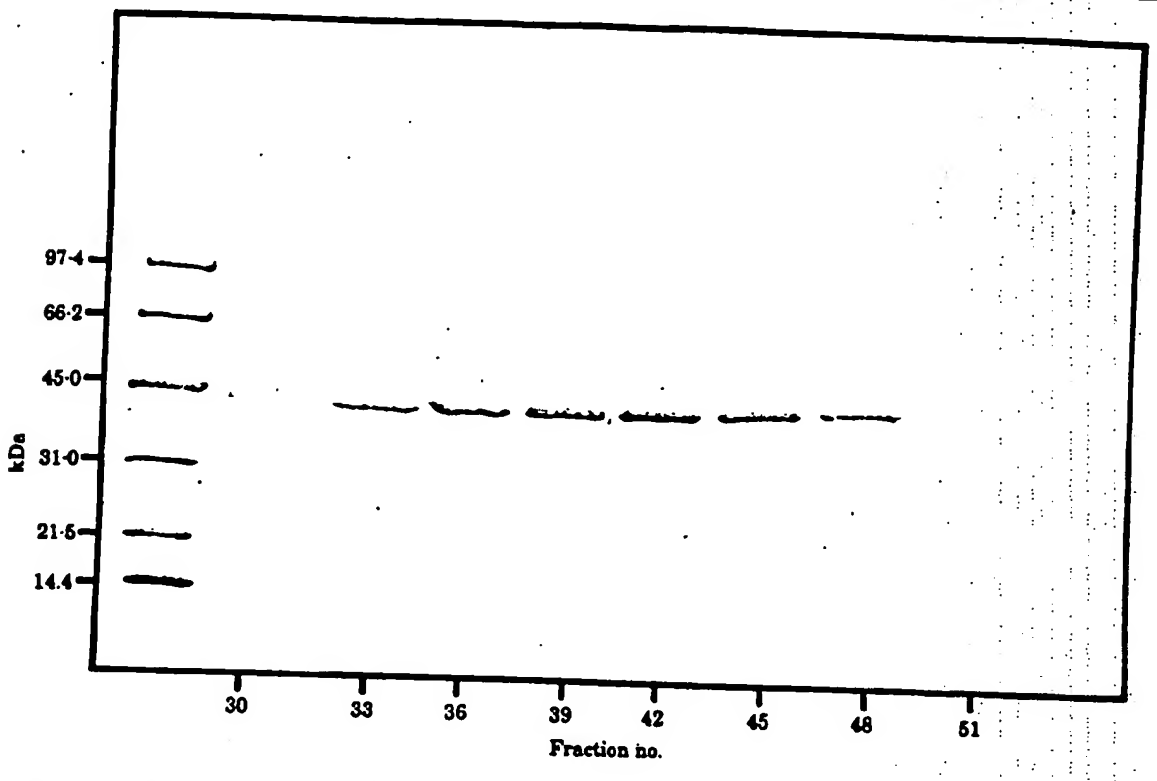

FIG. 2.—SDS-polyacrylamide gel analysis of fractions 30–51 from the heparin-Sepharose CL-6B chromatogram in Fig. 1. Five µl aliquots of the indicated fractions were combined with 25 µl SDS loading buffer, and electrophoresed on a 5–20% SDS-polyacrylamide gradient gel. Proteins were stained with Coomassie brilliant blue.

two patient groups and controls are shown in Fig. 5. The serum YKL-40 concentrations of patients with inflammatory rheumatic disease (median; lower quartile-upper quartile: 138 µg/l; 103–211 µg/l) was not statistically different ($P = 0.44$) from those in patients with OA (112 µg/l; 93–152 µg/l). Serum YKL-40 in both patient groups was significantly higher ($P<0.001$) than that of controls (50 µg/l; 36–64 µg/l).

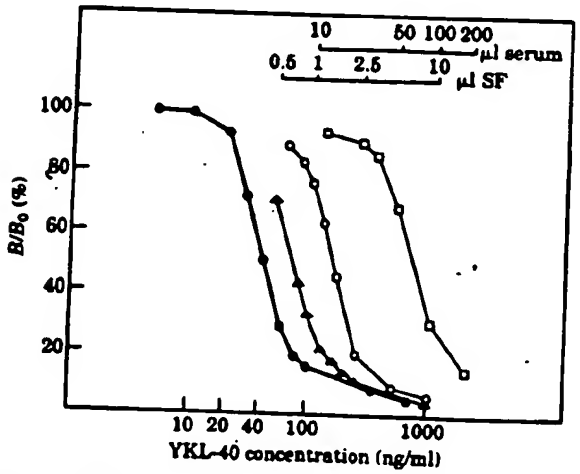

FIG. 3.—Radioimmunoassay for human YKL-40. Purified YKL-40 (●), bottom scale. Serum from a normal person (□), serum (○) and SF (△) from a patient with RA top scale.

YKL-40 in SF from patients with joint disease

The YKL-40 concentration in knee joint SF from the patients with inflammatory rheumatic disease (2210 µg/l; 1625–3040 µg/l) was not significantly different from the concentration in the patients with OA (1720 µg/l; 1270–1950 µg/l).

TABLE I

RELATIONSHIP BETWEEN SERUM AND SF CONCENTRATIONS OF YKL-40 AND OTHER BIOCHEMICAL MARKERS OF JOINT DISEASES

	Correlation coefficients	
	Serum YKL-40	SF YKL-40
Serum CRP	0.33•	0.31•
Serum IL-6	0.26	–0.10
SF IL-6	0.60••	0.47•
Blood Mø elastolysis	0.55••	0.20
SF Mø elastolysis	0.64••	0.58••
Serum PIIINP	0.49•••	0.13
SF PIIINP	0.02	–0.23
Clinical knee index	0.27	0.34•

Correlations are given as Spearman's ρ/P values. •$P<0.05$; ••$P<0.01$; •••$P<0.001$.

The correlations for serum CRP, serum and SF PIIINP, and clinical knee index are based from all 49 patients whose serum YKL-40 levels are shown in Fig. 5. The correlations for serum and SF IL-6 and for serum and SF Mø elastolysis are based on data from a 24 person subgroup of these patients.

IL-6, Interleukin-6; Mø elastolysis, elastolytic activity by live human monocytes/macrophages; PIIINP, the aminoterminal propeptide of type III procollagen.

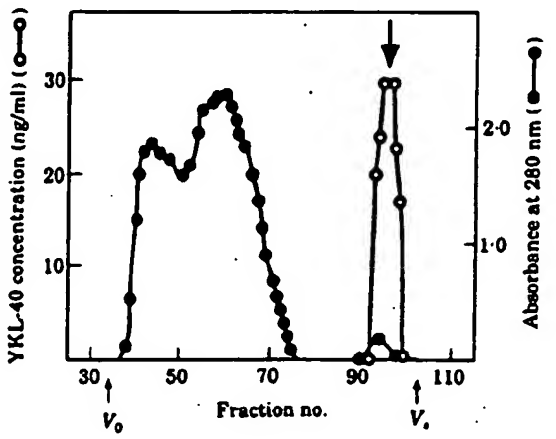

FIG. 4.—Gel filtration of serum from a patient with RA on a column of Sephacryl S-300 (see Methods). The arrow indicates the elution position of the purified human YKL-40 on this column. ●, A_{280}; O, YKL-40 determined by radioimmunoassay; V_0, void volume; V_t, salt volume.

Correlation between YKL-40 in serum and SF and other biochemical markers of inflammation and cartilage remodelling

The YKL-40 concentration in serum and SF in patients with inflammatory or degenerative joint diseases correlated significantly (Fig. 6) ($r = 0.55$; $P<0.001$) and the SF/serum YKL-40 ratio was high (15.0; 11.8–19.1). YKL-40 levels in serum and SF correlated significantly with serum CRP, SF fluid IL-6, and SF Mø elastolysis (Table I). Serum YKL-40 also correlated with blood Mø elastolysis and serum PIIINP. SF YKL-40 concentration correlated with a clinical index of knee inflammation. No correlation was found between YKL-40 in serum or SF and serum IL-6 and SF PIIINP levels.

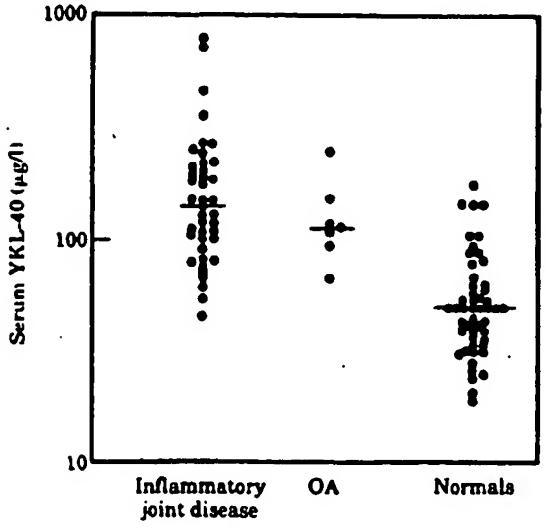

FIG. 5.—Individual YKL-40 concentrations in serum from patients with inflammatory or degenerative rheumatic diseases and from normal adults. The difference between YKL-40 concentrations in the two patients groups was not statistically significant ($P = 0.44$). Serum YKL-40 concentrations in the patients were significantly higher than in controls ($P<0.001$).

DISCUSSION

In the present study we have developed a new biochemical marker for joint disease, the measurement by RIA of the newly discovered protein YKL-40. With this assay we have shown that YKL-40 is present in serum and SF, and that the YKL-40 in these fluids is identical to purified YKL-40 in its molecular weight (Fig. 4) and in its ability to compete with purified YKL-40 tracer for binding to antibody (Fig. 3). The RIA for YKL-40 is based on a polyclonal antibody raised in rabbits against purified human YKL-40 and exhibits a sensitivity of 10 µg/l and has acceptable intra- and interassay variations.

Several of our findings indicate that serum YKL-40 levels reflect joint disease. The median level of serum YKL-40 in patients with inflammatory and degenerative joint disease is 2.5-fold higher than in healthy adults. In addition, the serum level of YKL-40 in the patient groups correlates with other biochemical indices of joint disease (Table I). Although it is not certain that all serum YKL-40 in fact arises from the joint, it is significant that SF YKL-40 levels are 15-fold higher than the levels in serum. The joint origin of some or all of serum YKL-40 is also supported by the positive correlation between serum and SF levels of YKL-40 in patients with joint disease (Fig. 6). Although it could be argued that the synthesis of YKL-40 by MG63 human osteosarcoma cells in culture is evidence for a bone origin of some serum YKL-40, it should be noted that normal adult osteoblasts do not secrete detectable YKL-40 in culture [2]. Furthermore the biopsy of MG63 metastases consists mainly of proliferating nodules of hypercellular cartilage [10]. In addition, unstimulated MG63 cells synthesize larger amounts of type III collagen than of type I collagen and secrete low to undetectable levels of alkaline phosphatase and of

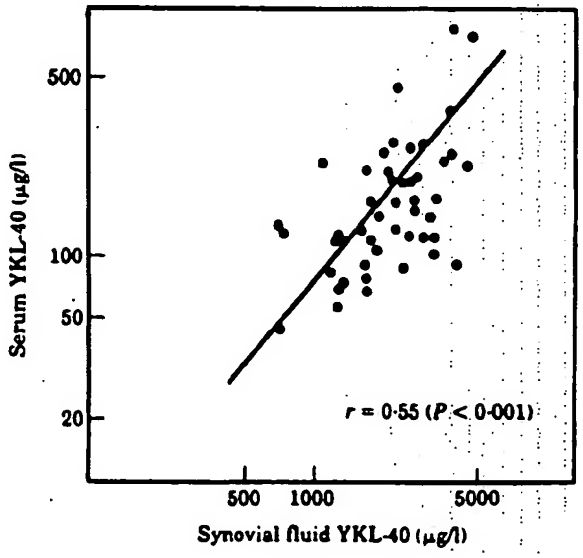

FIG. 6.—Correlation between YKL-40 in SF and serum in 49 patients with inflammatory or degenerative joint disease.

the bone specific protein BGP (osteocalcin) [11]. Since none of the patients used in this study had impaired liver or kidney function, it is not probable that elevated serum YKL-40 levels in patients with joint disease reflects reduced clearance of the protein from serum.

The positive correlation between SF YKL-40 levels and biochemical and clinical indices of joint disease (Table I) indicate that SF YKL-40 reflect joint disease. Further studies will be needed to determine if the elevated level of SF YKL-40 in joint disease is caused by increased synthesis of the protein by articular cartilage chondrocytes and synovial cells, or is due to decreased metabolic clearance of YKL-40 from the SF of the diseased joint.

Some insight into the physiological significance of elevated YKL-40 levels is provided by the biochemical indices of joint disease that correlate with YKL-40. The strongest significant correlation of serum and SF YKL-40 is with the elastolytic capacity of monocytes/macrophages, which mainly consists of human leukocyte elastase active at the cell surface [12]. This enzyme is capable of degrading proteoglycans [13] and collagens [14]. The second strongest correlation is to SF IL-6. IL-6 is one of the most effective regulators of inflammatory and immunological processes yet characterized [15] and is secreted by many cells including chondrocytes and synovial cells. High levels of this cytokine have been found in SF from inflammatory and degenerative arthropathies [16, 17]. Significant correlations are also found between YKL-40 and serum levels of PIIINP and of CRP. Serum PIIINP levels appears to reflect local inflammatory alterations in metabolism of type III collagen in synovium of patients with rheumatic diseases [18, 19]. CRP is the classical acute phase protein and although the *in vivo* function of CRP is still unknown, there is substantial evidence which indicates that it plays a significant biologic role during inflammation [20]. These correlations support a role for YKL-40 in the inflammatory response of the diseased joint, but it is not clear whether YKL-40 itself participates in the process of connective tissue destruction or is produced in response to tissue destruction, perhaps as a component of tissue repair. The lack of correlation between YKL-40 levels and some of the markers of inflammation as well as the relation to other markers suggest that a YKL-40 determination may provide new information on the pathological process in joint disease.

In conclusion we have described a novel biochemical marker for assessment of joint disease, the measurement by RIA of the recently discovered protein YKL-40 in serum and SF. Studies are currently in progress to determine the physiologic function and tissue distribution of YKL-40 in order to establish whether serum YKL-40 arises only from the joint and to understand the significance of serum YKL-40 in the assessment of joint disease.

NOTE ADDED IN PROOF

We have identified a possible function for YKL-40 based on protein sequencing data obtained after the original submission and assessment of this manuscript. Amino acid sequencing of a cyanogen bromide peptide derived from YKL-40 yielded the sequence LNTLKNRNPNLKTLLSVGG. This sequence has a 74% identity with chitinase A1 from *Bacillus circulans* (*J Biol Chem* 265:15659) and a 55% identity with a mouse protein secreted by activated peritoneal macrophages (unpublished; PIR Accession #S27879). A subsequent search using the published N-terminal sequence for YKL-40, YKLVCYYTSWSQYREGDGS, revealed a 42% identity with the *B. circulans* chitinase and a 58% identity with the mouse macrophage protein. Based on this information, we suggest that YKL-40 hydrolyses glycosidic bonds in an as yet unidentified macromolecule that is found in articular cartilage. Since chitin itself is not found in vertebrates, and since we could not demonstrate chitinase activity in YKL-40 using conventional assays, we speculate that divergent evolution of an ancestral chitinase altered the specificity of the vertebrate enzyme so that it now cleaves a different glycosidic linkage. While these new data fall short of providing proof of YKL-40 function, the serum YKL-40 data presented here can now be provisionally interpreted in light of the putative function of YKL-40 as a glycosidic bond hydrolyase.

ACKNOWLEDGEMENTS

This study was supported by grants from the Danish Medical Research Council (12-9528), Christian and Ottilia Brorsons Foundation and by USPHS grants AR 27029 and AG 07996. J. S. Johansen was the recipient of a research fellowship from the University of Copenhagen. We thank Dr M. Diamant for performing the IL-6 measurements and Dr L. T. Jensen for the PIIINP determinations.

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immunoelectrophoresis, these have become indispensable tools of the immunologist and immunochemist and have been applied in almost all fields of biological science. They are invaluable for analyzing complex mixtures of antigens and antibodies, for establishing that monospecific antisera contain only antibodies to the desired antigen, for detecting antibodies formed to impurities in the antigen used, and for qualitative detection and quantitative estimation of antigens and antibodies. Details are given by Oudin (this volume [9]).

Acknowledgments

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[2] Proteins and Polypeptides as Antigens

By PAUL H. MAURER and HUGH J. CALLAHAN

During the past two decades there has been a tremendous increase in the realization of the utility of antibodies directed against enzymes as tools in biochemical studies.[1,2] Antibodies against enzymes can be used (*a*) to detect and assay quantitatively the concentration of enzymes; (*b*) to concentrate and purify enzymes from dilute solutions and mixtures; (*c*) to study the active catalytic sites, multimolecular forms, and conformational structures of enzymes; (*d*) to localize enzymes in sectioned cells; (*e*) to study the appearance and modification of enzymes in the course of embryonic and phylogenetic development. Concomitantly in immunology there has been increasing knowledge concerning the many factors that can influence the multifaceted and complex sequence of events of the immune response beginning with the introduction of an antigen into a host (immunogen) to the formation of humoral antibody.[3] The goal of this chapter is not only to present information about techniques that have become available for producing antibody, but in so doing to make the inves-

[1] (B. Cinader, ed.). *Ann. N. Y. Acad. Sci.* 103, 493–1154 (1963).

[2] "Antibodies to Biologically Active Molecules" (B. Cinader, ed.). Pergamon, Oxford, 1967.

[3] See "Essential Concepts," discussed in: L. E. Hood, I. L. Weissman, and W. B. Wood, "Immunology," pp. 1–74. Benjamin-Cummings, Menlo Park, California, 1978.

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tigator aware not only of factors that might enhance antibody formation, but also of factors that might be operative in suppression of the immune response.[4]

As a rule biochemists and enzymologists work with limited amounts of purified materials. In addition to the usual biochemical methods, it should be realized that immunochemical techniques exist that allow one (*a*) to determine whether an enzyme preparation is indeed "pure" and (*b*) to use antibody against the enzyme to further purify the enzyme preparation via immunoadsorbent techniques.[5]

Knowledge of some immunological generalizations may help the investigator before he proceeds with the preparation of antibody. The ability of an animal to elicit an immune response depends upon complex interactions between the specific immunogen being presented to the host, the properties of the antigen, and the physiological state of the specific animal of choice. It is known that not all proteins or polypeptides can be immunogenic, i.e., can elicit an immune response under a standard set of conditions, and that the method by which the antigen is presented can influence the response. There also can be major differences in responses to the same macromolecule from species to species and among strains (or animals) within a specific species.[6] Nevertheless, it is known that by employing the correct "carrier" and conjugation procedure for the "nonimmunogen" there are ways of eliciting a response to almost any macromolecule.

The molecular weight and the complexity in structures of the macromolecule influence the nature of the immune response. In general, the greater the molecular weight and the more complex the protein or polypeptide structure, the greater the response that can be expected. From studies with synthetic polymers of amino acids, it has been learned that ordinarily one does not elicit significant responses against homopolymers of amino acids, and that decreased responses are obtained against high molecular weight polymers containing α-D- or γ-D-amino acids.[7,8]

Determination of the antigenic structures of proteins has posed a chemical challenge of enormous proportions for years.[9] Many investigators, therefore, have employed synthetic polymers of amino acids in im-

[4] D. H. Katz. "Lymphocyte Differentiation, Recognition, and Regulation." Academic Press, New York, 1977.
[5] I. Parikh and P. Cuatrecasas, *in* "Immunochemistry of Proteins" (M. Z. Atassi, ed.), Vol. 2 pp. 1–44. Plenum, New York, 1977.
[6] Reviewed in: "Immunogenicity—Physico-Chemical and Biological Aspects" (F. Borek, ed.). North Holland/American Elsevier, Amsterdam, 1972.
[7] P. H. Maurer, *Prog. Allergy* 8, 1 (1964).
[8] M. Sela, *Science* 166, 1365 (1969).
[9] "Immunochemistry of Proteins" (M. Z. Atassi, ed.). Vol. 1. Plenum, New York, 1977.

munochemical studies in the hope that information derived from these systems might be useful in the understanding of the immunochemistry of proteins. Although many data on these synthetic polymer systems have been accumulated in several laboratories, and polymers have contributed to elucidating aspects of the immune mechanism, the information gained from amino acid polymers has not always been helpful in understanding completely the immunochemistry or basis for the immunogenicity of proteins. Knowledge of the antigenic sites of protein antigens may help elucidate further not only the mechanisms of the immune response, but many immunological disorders at the molecular level.

Although the last decade has witnessed a great deal of activity investigating the immunochemistry of protein antigens,[3-9] so far the antigenic structure of sperm whale myoglobin[10] and of lysozyme have been completed.[11]

Factors Influencing the Immune Response

Animal Species

The more common animals used for immunization are rabbits, goats, sheep, chickens, horses, guinea pigs, and mice.[12,13] The eventual goals of having antibody against a specific protein or polypeptide may determine the specific species for immunization. Important considerations in choosing a species are the source and availability of the immunogen. As might be expected the larger animals require more antigen for the production of antibody, but when responding can yield more serum than others. Ordinarily one cannot obtain large amounts of serum from repeated bleedings of mice. However, there are techniques available for producing large amounts of ascites fluid rich in antibodies.[14-19] In addition, recent developments in the technology of the production of "hybridomas" of mye-

[10] M. Z. Atassi, *Immunochemistry* **12**, 423 (1975).
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[12] "Methods in Immunology and Immunochemistry" (C. A. Williams and M. W. Chase, eds.), Vol. 1. Academic Press, New York, 1967.
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[15] J. Munoz, *Proc. Soc. Exp. Biol. Med.* **95**, 757 (1957).
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[19] A. S. Tung, S. Ju, S. Sato, and A. Nisonoff, *J. Immunol.* **116**, 676 (1976).

loma cells fused with normal antibody-producing mouse spleen cells has afforded the production of larger amounts of antibody both *in vivo* and *in vitro*[20–24] (see below).

If one wants to increase the likelihood of obtaining a good response against most of the antigenic determinants in a protein, a species as phylogenetically removed as possible from the source (species) of the immunizing material should be injected. However, if a goal is to obtain antisera directed only against a few dissimilar structures or peptide sequences in an immunogen, then the same species should be injected. For instance, antibody against rabbit γ-globulin or mouse γ-globulin can be produced in rabbits and mice, respectively. However, depending on the genetic background of the host, the antibody produced can be directed against the minor "allotypic" structures.[25]

Generally, rabbits, sheep, goats, and horses produce much more antibody per milliliter of serum than do guinea pigs and mice. In addition, most of the antibodies obtained following hyperimmunization of rabbits, sheep, and goats precipitate with the homologous antigens, whereas not all of those produced in guinea pigs and mice precipitate easily, but tend to form soluble antigen–antibody complexes.

Genetic Factors

That many "genetic" factors govern immune responses to simple and complex synthetic polymers as well as to proteins such as lysozyme has been recently reviewed.[26] Immune responses of inbred strains of guinea pigs, mice, and rats to many immunogens are controlled by immune response genes present in the *major histocompatability complex* of the respective species. This has accounted for the unique finding of responders and nonresponders to polypeptides and proteins.[27] For instance, strain 2, but not strain 13, guinea pigs respond to the random copolymers

[20] G. Kohler and C. Milstein, *Nature (London)* **256**, 495 (1975).
[21] G. Kohler and C. Milstein, *Eur. J. Immunol.* **6**, 511 (1976).
[22] G. Galfre, S. C. Howe, C. Milstein, G. W. Butcher, and J. C. Howard, *Nature (London)* **266**, 550, (1977).
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[25] L. A. Herzenberg and L. A. Herzenberg, *in* "Handbook of Experimental Immunology" (D. M. Weir, ed.), 3rd ed., Vol. 3 Ch. 12, Blackwell, Oxford, 1978.
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$(Glu^{60}Ala^{40})_n$ and $(Glu^{60}Lys^{40})_n$ and the opposite pattern is noted with the polymer $(Glu^{50}Tyr^{50})_n$. Similar situations exist with inbred mice, i.e., mice of *H-2* haplotypes *a*, *b*, *d*, *f*, *k*, *s* respond to the random polymer $(Glu^{60}Ala^{40})_n$, but mice of haplotypes *p* and *q* do not. In addition a polymer or protein that is immunogenic in one species, need not be immunogenic in another species; i.e., mice do not respond to $(Glu^{60}Lys^{40})_n$ although guinea pigs and rabbits do.[28]

Because of the multigenic control of most immune responses, it is recommended that outbred animals be immunized first. Even with this precaution, it is likely that not all animals will respond similarly. However, the coupling of a poor immunogen either covalently or via electrostatic interactions with an immunogenic protein "carrier" can convert the nonimmunogen to a conjugate that is immunogenic in nonresponders and responders. Responses are then obtained to the complete conjugate, i.e., the carrier as well as the "haptenic" determinants. Some of the limitations to the above procedure are that the conjugation technique may alter the antigenic structure of the determinant and that "antigenic competition" between carrier and coupled macromolecule may occur if the carrier is very immunogenic.

Properties of the Antigen

Any consideration of the immunogenicity of a protein must take into account how its physiochemical properties can dictate the outcome of the immune response. We will consider only some of the intrinsic properties of antigens that are important in this respect; other properties will be considered in the section Methods of Immunization.

The state of aggregation of a protein has long been recognized as a factor involved in its immunogenic potential. Using bovine γ-globulin, Dresser[29] found that mice normally responsive to this protein were nonresponsive to preparations freed of particulate or aggregated material by centrifugation or column chromatography. Others[30] have demonstrated similar results in rabbits with human γ-globulin as the immunogen. In both cases the failure to mount an immune response seemed to be due to the induction of a tolerant (paralyzed) state by the deaggregated preparations.

Denaturation of protein antigens has been extensively studied over the past 70 years and in general has been shown to decrease immunogenicity relative to that of the native form. In addition the antigenic specificity of

[28] P. Pinchuck and P. H. Maurer, *J. Exp. Med.* 122, 665 (1965).
[29] D. W. Dresser, *Immunology* 5, 378 (1962).
[30] C. Biro and G. Garcia, *Immunology* 8, 411 (1965).

many native proteins is lost and new specificities are created. Several reviews[6,31,32] of this subject are available, and thus an extensive survey of the literature will not be attempted here, instead a few specific examples will be given that typify the results obtained.

Early studies demonstrated that denaturation of ovalbumin (OA) achieved by any of several means, e.g., heat, ultraviolet irradiation, sonication, alcohol, urea, or chemical modification resulted in a loss of reactivity with anti-native OA antibodies. In 1951 Maurer and Heidelberger[33] showed that upon deamination two fractions could be obtained from ovalbumin—one lightly deaminated (27–36%), which appeared not to be denatured, and another highly deaminated (40–80%), which was denatured and insoluble at its isoelectric point. The lightly deaminated preparation reacted completely with anti-native OA serum, and conversely antisera to this preparation reacted completely with native OA. The highly deaminated preparation, however, reacted only weakly with either antiserum. More recently, Jacobsen *et al.*[34] employing a series of chemically modified human serum albumins, have shown that a distinct relationship exists between the antigenicity of these proteins and the Stokes radius values as determined by gel filtration experiments. Using the Stokes radius as an indicator of unfolding, they have shown that an increase of approximately 0.5 nm results in a 50% loss in activity and an increase of 1.7 nm abolishes activity.

Analogous results have been obtained with reduced-carboxymethylated bovine serum albumin,[35] performic acid-oxidized ribonuclease,[36] reduced-carboxymethylated lysozyme,[37] and several other proteins.

The studies described above, as well as numerous others, have led to the conclusion that protein antigens may contain two general classes of determinant structures, namely, sequential and conformational. Sequential determinants would be those occurring in a linear conformation, as in the unfolded form of a protein, and conformational determinants would be those that are recognized by their homologous antibodies only when they occur in a particular conformation. The latter class would include determinants formed from amino acids that are located at distant points in the

[31] E. A. Kabat, *in* "Kabat and Mayer's Experimental Immunochemistry," 2nd ed. Thomas, Springfield, Illinois, 1961.
[32] M. Reichlin, *Adv. Immunol.* p. 29. Academic Press, New York, 1975.
[33] P. H. Maurer and M. Heidelberger, *J. Am. Chem. Soc.* 73, 2076 (1951).
[34] C. Jacobsen, L. Funding, N. P. H. Moller, and J. Steengard, *Eur. J. Biochem* 30, 392 (1972).
[35] E. J. Goetzl and J. H. Peters, *J. Immunol.* 108, 785 (1972).
[36] R. K. Brown, *J. Biol. Chem* 237, 1162 (1962).
[37] J. D. Young and C. Y. Leung, *Biochemistry* 9, 2755 (1970).

peptide chain but come into proximity with each other when a particular conformation is achieved. An elegant demonstration of conformational effects has been reported by Arnon and Sela.[38] They were able to isolate a 20-amino acid peptide (Cys^{64}-Leu^{83}) from peptic digests of egg white lysozyme, which was joined at residues 64 and 80 by a disulfide bridge, thus forming a "loop". This "loop" peptide was conjugated to a synthetic branched polypeptide and then used to immunize rabbits. Antibodies immunospecifically isolated from these sera, or from sera of animals immunized with lysozyme, react with the "loop" peptide, but only poorly or not at all with the open chain form produced by reduction and carboxymethylation, indicating the requirement for a particular three-dimensional structure in the antigen to bind with antibody.

It also seems likely that proteins displaying quaternary structure have unique determinants that can be considered "conformational." Thus Reichlin[32] has shown that, in complement fixation tests, antisera against methemoglobin reacted better with oxyhemoglobin than with deoxyhemoglobin. This is most likely due to the known difference in quaternary structure between the two proteins. In addition, the isolated α and β chains, which were inactive with the antiserum, could be rendered active by recombination with the appropriate chains from a different species. With myoglobin similar results were obtained, i.e., antisera to myoglobin detected differences between the heme-containing protein and the heme-free protein. It appears at present that most determinants in globular proteins are of the conformational type whereas one finds both types in structural proteins such as collagen.[39]

There are numerous properties, other than those mentioned, that are associated with antigens and would affect their ability to induce antibodies or our ability to detect these antibodies. We will consider only four here, since they are more applicable to proteins and polypeptides: accessibility of determinants, complexity of amino acid composition, molecular weight, and effect of ions. It is well documented that groups that function as antigenic determinants are those that are exposed to solvent. This has been elegantly demonstrated by Sela's group[8] with branched-chain synthetic polypeptides. They found that when tyrosine and glutamic acid residues were attached in a linear fashion to poly(DL-alanine), and the alanine is then linked to poly(L-lysine) as a branch, antibodies were formed against the terminal glutamic acid and tyrosine positions (Fig. 1). However, if alanine was placed in the terminal position and linked to the poly-

[38] R. Arnon and M. Sela, *Proc. Natl. Acad. Sci. U.S.A.* 62, 163 (1969).
[39] M. Crumpton, *in* "Defence and Recognition" (R. R. Porter, ed.), MTP Int. Rev. Sci. Ser. I, Vol. 10. Butterworth, London, 1973.

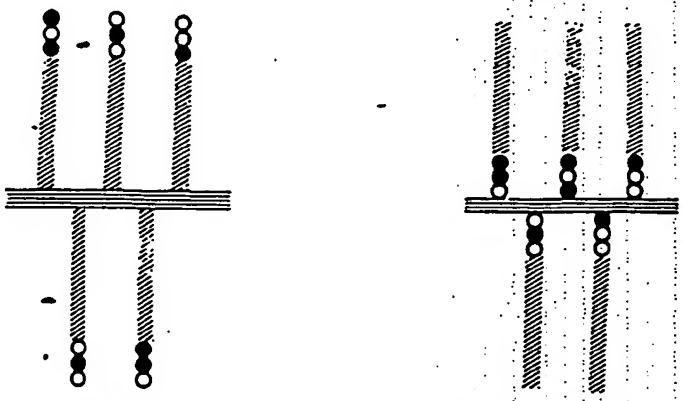

FIG. 1. A multichain copolymer in which L-tyrosine and L-glutamic acid residues are attached to multi-poly(DL-alanyl)-poly(L-lysine). *Left:* Tyrosine and glutamic acid located in terminal positions. *Right:* Tyrosine and glutamic acid positioned internally. Horizontal lines, poly(L-lysine); hatched area, poly(DL-alanine); ●, L-tyrosine; ○, L-glutamic acid. From M. Sela.[9]

lysine through the glutamic acid or tyrosine, then most of the antibodies were alanine specific. These data strongly argue that, in order to be antigenic, determinants must be exposed to the environment. This concept is supported by Atassi's studies[10] with myoglobin. Atassi was able to identify five antigenic regions in this protein, and all were located in solvent-accessible regions, in this case on the surface of the molecule.

It is generally accepted that a relationship exists between the structural complexity of a compound, i.e., the variety of its components, and its ability to induce an immune response. For example, homopolymers of amino acids by themselves are very poor antigens,[40-42] however, when used in a complex (e.g., with phosphorylated serum albumin), they induce normal levels of antibodies.[43] The same effect can be accomplished by the introduction of a second or third different amino acid.[44] Analogous situations exist in some naturally occurring macromolecules. Thus, the low level of antibodies induced by gelatin could be greatly elevated by the introduction of tyrosyl residues.[45,46] At the present time, we cannot explain

[40] S. B-Efraim, S. Fuchs, and M. Sela, *Immunology* 12, 573 (1967).
[41] P. H. Maurer, *Proc. Soc. Exp. Biol. Med.* 96, 394 (1957).
[42] D. Subrahmanyam and P. H. Maurer, *Fed. Proc.* 18, 600 (1959).
[43] H. Van Vunakis, J. Kaplan, H. Lehrer, and L. Levine, *Immunochemistry* 3, 393 (1966).
[44] P. H. Maurer, *Ann. N. Y. Acad. Sci.* 103, 549 (1963).
[45] M. Sela and R. Arnon, *Biochem. J.* 75, 91 (1960).
[46] M. Sela, B. Schechter, I. Schechter, and F. Borek, *Cold Spring Harbor Symp. Quant. Biol.* 32, 537 (1967).

this phenomenon, although it has been suggested that cooperation between T and B cells in the immune response requires that different specificities exist within the antigen.[47]

In considering the relationship of an antigen's molecular size to its capacity to stimulate an immune response, one must examine both humoral and cellular immunity, since their requirements are somewhat different. In general there is a direct relationship between molecular weight and the ability to induce an immune response in high molecular weight compounds. Thus, many polymers, e.g., flagellin,[48] dextran, and pneumococcal polysaccharide,[49] demonstrate increased humoral and cellular responses with increased size, but this is by no means absolute, since several synthetic polypeptide antigens of the same overall composition but widely different molecular weights have induced the same amount of antibody.[50]

Attempts to determine the minimum size necessary to evoke a response have been much more definitive. Schlossman *et al.*[51] showed, by using a homologous series of α-DNP-oligo(L-lysine) compounds ranging in size from the tetramer to the nonamer, that a chain length of seven units was the smallest size that could induce humoral and cellular immunity in guinea pigs. Smaller oligomers were ineffective. These results were confirmed by Stupp *et al.*[52] using ε-DNP-oligo(L-lysine) compounds, but it was also discovered that the incorporation of oligopeptides into Freund's adjuvant made significant differences in response patterns. Thus, mono-ε-DNP-oligo(L-lysine) compounds, containing as few as two lysine residues, when emulsified with mycobacteria in complete Freund's adjuvant, were capable of stimulating anti-DNP antibody production but not delayed hypersensitivity reactions. No antibody was produced when incomplete adjuvant or saline was used. As an explanation it was suggested that the mycobacteria in the adjuvant formed complexes with the positively charged peptides and thus acted as a carrier.

Although it has been known for many years that high concentrations of many salts can inhibit antigen–antibody interaction or dissociate im-

[47] J. Goodman, *in* "The Antigens" (M. Sela, ed.), Vol. 3, pp. 127–183. Academic Press, New York, 1975.
[48] M. J. Becker, H. Levin, and M. Sela, *Eur. J. Immunol.* 3, 131 (1973).
[49] K. Jann and O. Westphal, *in* "The Antigens" (M. Sela, ed.), Vol. 3, pp. 1–110. Academic Press, New York, 1975.
[50] T. J. Gill, III, H. W. Kunz, and D. S. Papermaster, *J. Biol. Chem.* 242, 3308 (1967).
[51] S. F. Schlossman, S. Ben-Efraim, A. Yaron, and H. A. Sober, *J. Exp. Med.* 123, 1083 (1966).
[52] Y. Stupp, W. E. Paul, and B. Benacerraf, *Immunology* 21, 583 (1971).

mune complexes, it was only recently discovered that in some systems physiological concentrations of specific ions are necessary to affect interaction. Using a synthetic polypeptide composed of 60% glutamic acid, 30% alanine, and 10% tyrosine (GAT), Maurer *et al.*[33] found that some animals (particularly sheep) respond to immunization with the production of two distinct populations of antibodies—one that reacts with the antigen only if divalent cations are present, and another having no such requirement. It was shown with several sheep and rabbit antisera that addition of a chelating agent (EDTA) with the antigen prevented precipitation of 10–90% of the antibody. These experiments led to the isolation of the antibodies and subsequently to a physicochemical explanation of the cation's role. Liberti *et al.*[34] showed that cations, especially calcium, neither affect the antibody itself nor enhance precipitation of preformed antigen–antibody complexes. They did show that calcium affects the antigen by decreasing intrinsic viscosity, increasing $s_{w,20}$ and changing the optical rotatory dispersion pattern. Bivalent cations probably induce conformational changes in the antigens, perhaps by bridging the carboxyl groups of glutamic acids, leading to the creation of "new" antigenic determinants. In addition to synthetic polypeptides, this cation effect has been found with polysaccharide and protein antigens. Approximately 10–20% of rabbit antibodies raised against two pneumococcal polysaccharides (type III and type VIII) had a calcium requirement for interaction with the homologous antigen.[35] Not surprisingly, both polysaccharides contain glucuronic acid residues in their determinant structures. Favre and Vollotton[36] found 7 of 14 rabbits immunized with angiotensin II had antibodies that bound the antigen maximally in the presence of calcium. More recently[37] calcium requiring anti-human serum albumin antibodies from both rabbits and sheep have been isolated and characterized.

Methods of Immunization

Very detailed and helpful techniques dealing with the preparation of immunogens for immunization and the use of various vehicles and methods for immunization are presented in "Methods in Immunology and Immunochemistry,"[12] Volume I, and in the "Handbook of Experimental Immunology."[13]

[33] P. H. Maurer, L. G. Clark, and P. A. Liberti, *J. Immunol.* 105, 567 (1970).
[34] P. A. Liberti, H. J. Callahan, and P. H. Maurer, *Adv. Exp. Med. Biol.* 48, 161 (1974).
[35] H. J. Callahan and P. H. Maurer, *Immunol. Commun.* 4, 537 (1975).
[36] L. Favre and M. B. Vallotton, *Immunochemistry* 10, 43 (1973).
[37] M. E. Frankel, H. J. Callahan, and P. A. Liberti, *Fed. Proc.* 36, 1286 (1977).

In dealing with limited amounts of valuable protein or polypeptide one should use techniques that might enhance the immune response. Although responses can be obtained against either solutions of soluble antigens or suspensions of particulate antigens, better responses are elicited with the proper use of "adjuvants." In principle, the adjuvants allow use of much less immunogen for some of the following reasons. Not all of an immunogen administered to a host persists long enough to become an effective stimulator for antibody formation. Adjuvants increase the persistence of antigen in the host and can protect the antigen from degradation by the usual proteolytic enzymes. This can allow more antibody-forming cells to be exposed to the limited amount of antigen. Particulate materials are more immunogenic, and therefore it is advisable, if possible, to aggregate the protein artificially providing the procedure does not change the conformation or biological activity. Aggregates of human γ-globulin and bovine serum albumin are immunogenic whereas the "monomeric" form of these proteins can be tolerogenic.[58] Macromolecules that are highly charged, and even those that are nonimmunogenic, can be made to react with such carriers as methylated bovine serum albumin or phosphorylated bovine serum albumin. The charge interaction leads to an insoluable aggregate, which can be immunogenic. This technique has been successful for producing antibody against charged macromolecules, such as DNA,[59] polynucleotides,[60] polyglutamic acid.[42] However, one has to be aware that the reaction might lead to changes in the structure of the immunogen or to masking or creation of new immunogenic determinants.

Processing of an antigen by macrophages is an important aspect of the immune response, and therefore any procedure that enhances the uptake by macrophages before presentation to the lymphocytes augments the response. Adjuvants allow concentration of the antigen onto a particulate carrier so that the amount of antigen administered per unit volume is increased, the immunogen can be localized in specific areas for long periods of time, and local destruction and elimination of antigen is retarded.

The commonly recognized adjuvants are remarkable for their diversities. Soluble immunogens can be adsorbed onto the following kinds of inorganic suspensions: alumina cream, aluminum phosphate, and aluminum sulfate. Adsorption onto organic carriers such as blood, charcoal, calcium alginate, or polyacrylamide gels has also enhanced responses. A Maalox

[58] W. O. Weigle, "Natural and Acquired Immunologic Unresponsiveness." Cleveland World Publ., Cleveland, Ohio, 1967.

[59] O. J. Plescia, W. Braun, and N. C. Palczuk, *Proc. Natl. Acad. Sci. U.S.* **52**, 279 (1964).

[60] "Nucleic Acids in Immunology" (O. J. Plescia and W. Braun, eds.). Springer-Verlag, Berlin and New York, 1968.

[$Al(OH)_3$] suspension has also been used in conjunction with the addition of bacteria, such as *Bordetella pertussis*.

The most popular and successful adjuvants have been the water in oil emulsions developed by Freund. The basic ingredients of light mineral oil (Bayol) and emulsifying agents mixtures such as Arlacel (A or C) are available commercially. The reagents are emulsified with either solutions or suspensions of the immunogen (incomplete Freund's adjuvant). The addition of mycobacteria (*Mycobacterium butyricum*, *M. tuberculosis*) in small amounts to the suspension (complete Freund's adjuvant) leads to a further enhancement of the immune response. This has been attributed to the increased local inflammatory response caused by the mycobacteria.[61]

The well mixed and stable emulsion is injected either intraperitoneally, intradermally, or in the footpads of the host. If complete Freund's adjuvant has been used for the first injection, the secondary injections should not contain the mycobacteria, as further immune responses against the mycobacteria can be detrimental to the host and also lead to enhanced inflammatory responses. There are some additional advantages to using the complete adjuvant. The class of antibody formed is sometimes altered, leading to precipitating antibody, and ascites fluid can be formed following intraperitoneal injections in mice. Although it is not always predictable mice can produce large amounts of this fluid, which has concentrations of antibody almost equal to that found in the serum. On the negative side the investigator should be aware that the emulsion might destroy or mask some of the antigenic determinants of labile antigens.

As a matter of convenience the immune response is divided into two stages. The primary response, resulting from an initial interaction with antigen, and the secondary response, resulting from subsequent contact with the same antigen. Quite often it is difficult to clearly delineate between the two: for example, an animal may have seen the antigen previously (particularly microbial antigens) or may have been exposed to cross-reacting antigens: however, for the present these factors will be discounted.

The primary immune response is characterized by the appearance, within a few days of 19 S (IgM) antibodies. This is followed by a decline in 19 S levels and a rise in 7 S (IgG) antibody levels, which in general is dose dependent: for example, a good immunogen given in very low doses elicits little 7 S antibody, whereas in high doses quite significant amounts are formed. This phenomenon is nicely illustrated in the work of Uhr and Fin-

[61] R. G. White, *in* "The Immunologically Competent Cell: Its Nature and Origin" (G. E. Wolstenholme and J. Knight, eds.). Churchill, London, 1963.

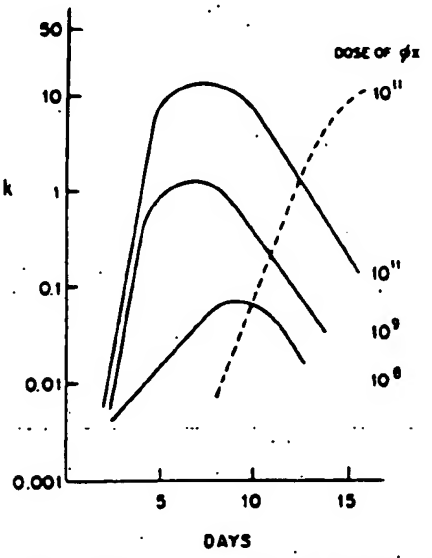

FIG. 2. The primary 19 S(—) and 7 S (---) antibody responses to ϕX174 bacteriophage (ϕX) in the guinea pig. Representative responses to intravenous injections of 10^{11}, 10^{9}, or 10^{8} phage particles are shown. From Uhr and Finkelstein.[62]

kelstein.[63] Using the bacteriophage ϕ×174, a very potent antigen, they showed that injection of 10^{8} of 10^{9} particles led to good IgM response with no IgG production. However, administration of 10^{11} phage led to high IgM titers followed several days later by a large increase in IgG (Fig. 2). After about 2 weeks the IgM response had fallen off and the antibody was mostly of the IgG class.

There has been some question as to whether soluble protein antigens induce the same pattern of primary response as particulate ones, and it now appears that they do, although the length of time between injection of antigen and appearance of antibody (lag phase) may be delayed.

The secondary response results from the readministration of antigen at a later time and is characterized by a rapid increase in antibody levels consisting mainly of IgG, but with some transient IgM. The most striking effect observed is the great increase in total serum antibody levels over that obtained in the primary response[63] (Fig. 3). After 2–3 weeks there is a

[62] J. W. Uhr and M. S. Finkelstein, *Prog. Allergy* 10, 37 (1967).
[63] J. W. Uhr, M. S. Finkelstein, and J. B. Bauman, *J. Exp. Med.* 115, 655 (1962).

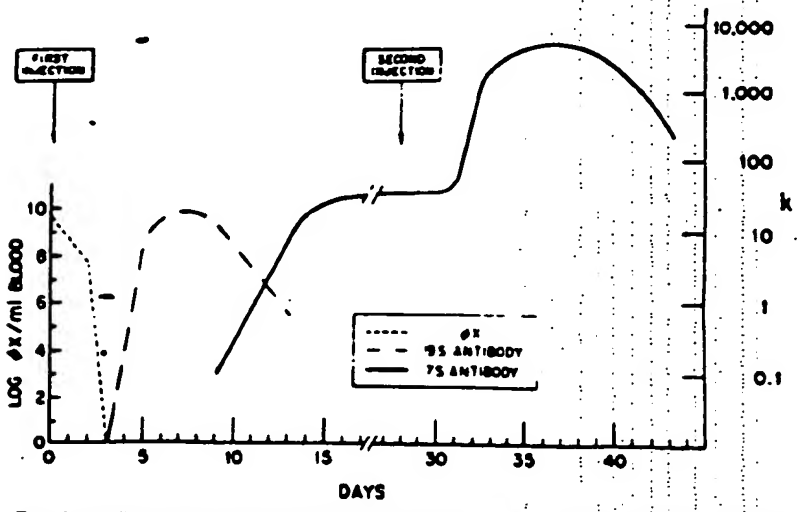

FIG. 3. Antibody response to φX174 in the guinea pig after two intravenous injections. ---, φX174 bacteriophage (φX); – –, 19 S antibody response; —, 7 S antibody response.

rapid decline in the IgG level until antibody concentrations reach a plateau, at which they may persist for weeks or months.

The dose of antigen used for immunization, in addition to modulating the classes of immunoglobulin formed, can also have a profound effect on the ability of the animal to produce antibody at all. A refractory state, called tolerance, can be induced with most proteins having a low or moderate molecular weight (e.g., serum proteins) provided that the amount of antigen administered is within a given range. This tolerant state may be defined as the inability or diminished ability of an animal to react to a normally immunogenic material that has been induced by previous administration of the same material. It has shown that tolerance can be achieved within two distinct zones of antigen dosage. High zone tolerance is achieved when quantities of antigen much greater than the optimum immunizing dose are presented. Mitchison,[64] for example, induced tolerance to bovine serum albumin in mice by injection of 10 mg three times per week for 10 weeks. This type of tolerance is readily achieved with weaker immunogens (e.g., soluble proteins), but it is difficult to induce with potent immunogens because the quantities needed tend to be toxic or impractical to use.

[64] N. A. Mitchison, *in* "Immunogenicity" (F. Borth, ed.), p. 87. North-Holland Publ., Amsterdam, 1972.

Low zone tolerance is induced with subimmunogenic amounts of antigen, approximately 1 μg or less in the mouse. Repeated administration of antigen is usually necessary to induce or maintain the tolerant state, or both.

There is still no foolproof method for choosing the route of administration of antigen in order to evoke a humoral response. In general, weaker immunogens are used with Freund's adjuvant and given intramuscularly or subcutaneously. With protein antigens the intravenous route is often used in tolerance induction; the intradermal route, minus adjuvant, is employed to induce delayed hypersensitivity.[65] A final consideration in regard to antigen dosage is that of the amounts, affinity, and specificity of the antibodies produced during the immune response. Siskind *et al.*[66] have shown that in rabbits immunized with DNP–bovine γ-globulin, high doses of antigen (50 mg) resulted in a rapid increase in serum antibody levels followed later in the immune response by a decrease and plateauing at low levels. When a low dose (0.5 mg) was used, the response began slowly but increased with time and eventually exceeded by threefold the high-dose level. These authors also showed a progressive increase in antibody affinity with time after immunization. Although all the doses used induced this effect, the increase was much greater with the lower ones.

After a consideration of the cellular events involved in antibody production, it was suggested that these results could be interpreted as follows: the large doses of antigen injected might induce tolerance in "high affinity" cells resulting in a decrease in the amount and affinity of antibody produced. An alternative possibility is that large doses of antigen would favor differentiation of cells for antibody production resulting in the rapid appearance of high antibody titers but with a concomitant depletion in proliferating cells, thus limiting the response. Lower concentrations would give a more sustained and eventually greater response: In brief, the current explanation for an affinity increase is that after initial immunization a number of cell types, of both high and low affinity, are stimulated. Later, as the concentration of available antigen decreases owing to metabolism, only those cells that can bind antigen strongly (high affinity) are stimulated. This theory is also used to explain the generally observed increase in cross-reaction of antibodies that occurs with time during the immune response, by noting that, as affinities increase, determinants that would not have bound well with early (low affinity) antibodies now can be bound well enough to be detected.

[65] J. W. Uhr, *Physiol. Rev.* 46, 359 (1966).
[66] G. W. Siskind, P. Dunn, J. G. Walker, *J. Exp. Med.* 127, 55 (1968).

Typical Protocols for Antibody Production

In Vivo Techniques

After consideration of the many factors that influence the immune response and the availability of material, most investigators prefer to incorporate the antigen in Freund's complete or incomplete adjuvant. The preparation for immunization need not be absolutely homogeneous, but the antigen to be used in the assay of the immune response should be highly purified. At times the purity of the immunogen may be important, as one might encounter the phenomenon of antigenic competition, wherein the response to the impurities might mask the responses against the putative purified enzyme.

For immunization of large animals, such as rabbits, sheep, or goats, small to moderate amounts of antigen should be used (0.1–1.0 mg per kilogram of body weight). A typical protocol would employ 5 or 6 rabbits injected either in the footpads or intramuscularly into multiple sites with about 1–5 mg in complete Freund's adjuvant in a total volume of 0.25–0.5 ml. With smaller animals, such as mice and guinea pigs, microgram amounts (1–100 μg in 0.02–0.2 ml) are injected in the footpads subcutaneously or intraperitoneally. The animals are bled weekly for 4–6 weeks beginning about 3 weeks after the immunization; the sera are separated and tested qualitatively (see below) for the production of antibody. Rabbits and larger animals can be bled from the ear vein or via jugular vein puncture. With mice, sera can be obtained via retroorbital tappings of blood.

Several techniques have been developed for producing ascites fluid in mice.[15–19] In addition to the original adjuvant technique, a modification used in our laboratory involves injecting the antigen in complete Freund's adjuvant intraperitoneally followed by an injection 3 days later of 0.5 ml of pristane.[19] Mice are boosted 7–10 days later and again after another 7–10 days. Another effective method is to administer intraperitoneal injections of Sarcoma 180 cells[18] subsequent to several intraperitoneal injections of complete Freund's adjuvant. Distension of the abdomen is indicative of ascites development. The amount of ascites fluid that can be obtained at each tapping varies from 0.25 ml to 20 ml.

If the level of antibody is rising significantly, or there is no response at all, booster injections need not be given. It is important to have intervals between injections and to be careful not to reinject if there are high levels of antibody. When the antibody level is rising slowly or has decreased, it is beneficial to reinject the animals with incomplete Freund's adjuvant or the antigen solution intraperitoneally. In the presence of high levels of antibody, anaphylactic shock can ensue in mice or rabbits.

Pooling of sera from different outbred animals is not recommended, as reactions to different determinants might have occurred in different animals. In addition, if the immunizing preparation is not absolutely pure, it is conceivable that some animals might have responded very well to the impurities. Changes do occur in the properties of the antibody produced over a period of time. In general high affinity antibody follows immunization with low doses of antigen, and the best sera in rabbits can be obtained about 3–5 months after immunization. Although not always predictable, animals that respond early after immunization usually produce the best antibody.[67]

In Vitro Techniques

A unique and revolutionary adaptation of cell hybridization techniques to the construction of myeloma-like cell lines producing *monoclonal antibodies* with desired reactivities has revolutionized the approach to the production of immunospecific reagents.[20–24] Large amounts of specific antibody can be obtained after hybridization of lymphoid cells from an appropriately immunized donor (mouse) with cells from a mouse myeloma that has been adapted to growth in culture. Although the normal *in vivo* immune response to complex antigens leads to a very heterogeneous population of antibody molecules directed against many determinants, with the hybridoma technique each hybrid clone theoretically produces a single species of antibody specific for a single antigenic determinant. A recent symposium on cell hybridomas[23] and other publications[20–24] discuss in depth both the technology and the applicability of the procedure.

Essentially the protocol (Fig. 4) involves hybridizing spleen cells from a hyperimmunized donor with cells from an *in vitro* adapted enzyme-deficient myeloma. A neoplastic cell line used for producing fusions is X63-AG8, a clonal line of myeloma MOPC21 that has been adapted to growth *in vitro*, in 8-azaguanine, and lacks the enzyme hypoxanthine guanine phosphoribosyltransferase (HGPRTase) required for rapid growth in tissue culture medium containing hypoxanthine, aminopterin, and thymidine (HAT medium). The fusing agent is polyethylene glycol (PEG) of a specific concentration and molecular weight. After fusion (hybridization) the cells are cultured in HAT medium. Before subculturing, the supernatants of the initial hybrid cells can be assayed for antibody production by a number of sensitive techniques that allow one to determine which clones from a complete spleen are involved in antibody production and therefore are worth further subculturing. Once established the clones can

[67] G. W. Siskind and B. Benacerraf, *Adv. Immunol.* 10, 1 (1969).

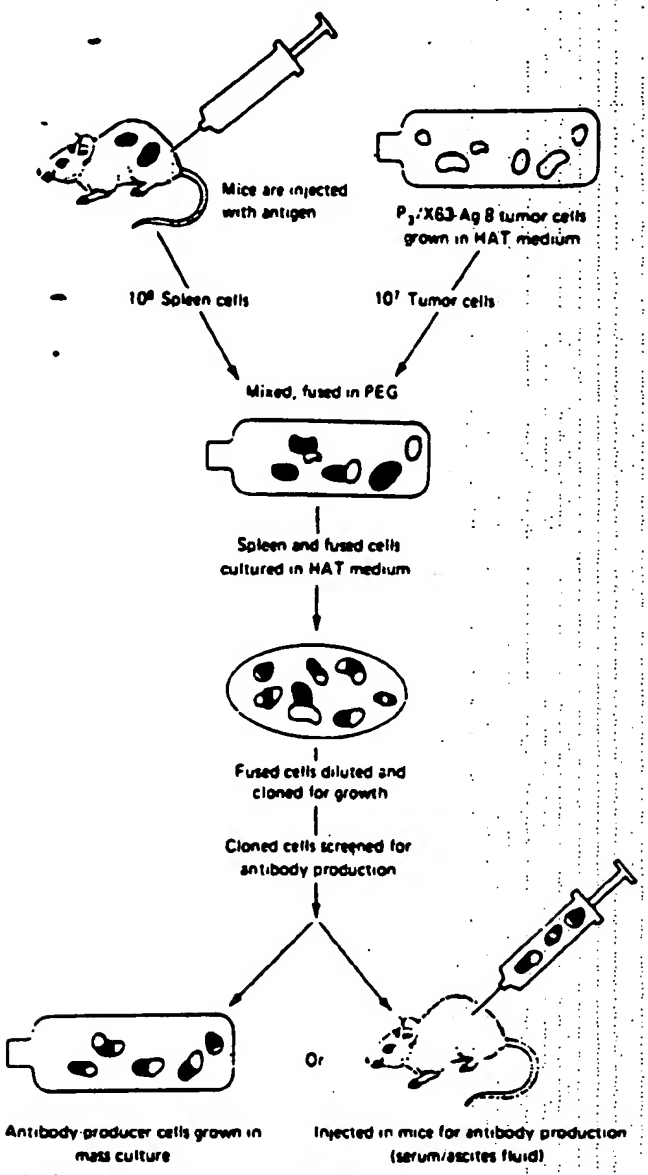

FIG. 4. Multistep methods for eliciting specific antibody-producing cells (hybridomas). HAT, hypoxanthine, aminopterin, and thymidine; PEG, polyethylene glycol.

be kept growing either in culture or *in vivo* for many months. In the *in vivo* technique the culture-grown antibody-forming clones are injected into pristane primed mice. Within a few weeks the ascites-containing fluid that appears has monoclonal antibody.

When grown in mass culture, antibody concentrations can reach about 50 μg/ml. Immunoadsorbent techniques can then be used to concentrate the specific antibody. In a number of situations where the hybridized cells have been injected *in vivo*, concentrations of 5–20 mg per milliliter of antibody have been produced.

In the specific area of enzymology, success has been achieved in producing clones against human alkaline phosphatase, hen egg lysozyme, and horseradish peroxidase.[68] A major advantage of this technique is that one can obtain monospecific antibodies directed against the immunogenic determinants of the enzyme, some of which might be against the active site of the enzyme.

Techniques for Assaying for Antibody

Initially a qualitative test, then a semiquantitative and, if deemed useful, a quantitative estimation of antibody can be performed.[12–14] The table presents the sensitivity of some of the *in vitro* serological tests that can be used. (The advantages and disadvantages of the different techniques will be discussed by other authors.) When detecting immune responses in different species, one should reckon with the fact that not all antibody systems lead to a precipitation reaction. Rabbit sera containing precipitating

COMPARISON OF MINIMAL CONCENTRATION OF ANTIBODY DETECTABLE BY SPECIFIC TEST

Immunological test	Antibody detectable (μg N/ml)
1. Fluid precipitation	
Interfacial (ring) test	20–30
2. Gel precipitation	
Double diffusion (Ouchterlony)	3–15
Single diffusion (Oudin)	10–100
3. Hemagglutination	
Passive (indirect)	0.001–0.03
4. Radioimmunoassay	0.001–less

[68] See references to hybridomas produced against enzymes *in* "Lymphocyte Hybridomas" (F. Melchers, M. Potter, and N. L. Warner, eds.). *Curr. Top. Microbiol. Immunol.* **81**, 19–22 (1978).

antibody can be screened by both the agar diffusion techniques and/or reactions in liquid medium. It is best first to test the sera by precipitation in gel techniques. Although this is a *secondary reaction*, based upon complex interactions of antigen–antibody complexes following the initial interaction, a positive reaction (band formation) is indicative of significant concentrations of antibody. The ring or interfacial test involves carefully overlaying a solution of antibody and its dilutions with antigen so that a sharp interface is formed. Diffusion occurs between the two components until an optimal ratio (equivalence) for precipitation of the complexes is established.

The gel diffusion tests involve carrying out the reactions between antigen and antibody in a semisolid medium. There are many modifications of this technique, i.e., Preer, Ouchterlony, and Oudin, all of which lead to "band" formation.[14] In addition to detecting antibody qualitatively and estimating the approximate equivalence ratio needed for optimal precipitation between antigen and antibody, the agar diffusion techniques can indicate the number of antigen–antibody systems that might be present, providing that the diffusion effects due to temperature and concentration are controlled.

A disadvantage of the reaction in liquid medium resides in the fact that if the concentration of antigen added is too high, soluble antigen–antibody complexes will form in antigen excess and it may appear as though there is no significant amount of antibody present. Therefore varying concentrations of antigen must be added to both undiluted and varying dilutions of serum in a checkerboard fashion.

Although there are many *in vitro* and *in vivo* methods for estimating antibody in serum or other fluids, only the quantitative precipitin reaction gives a reliable and accurate measure of antibody in absolute weight units. When a proper assessment of the many factors influencing the reaction is made and rigorous procedures of quantitative analysis are employed in analyzing the washed antigen–antibody specific precipitate, quantitive antibody values are obtained.[31]

In addition to the direct reaction of the antigen with antibody, techniques are available for performing "indirect" reactions. Antigens can be coupled chemically or via a "tannic acid" procedure to red blood cells. The antigen-coated erythrocytes in the presence of specific antibody then agglutinate (clump) as do red blood cells in the presence of antibody to the red blood cells (hemagglutination).

Rather than measuring the capacity of an antiserum to *combine* with antigen, all the above-mentioned antibody tests measure the capacity of an antiserum to produce secondary effects, i.e., precipitation and complement fixation, following the primary antigen–antibody interaction.

Measuring the antigen binding capacity rather than the absolute amount of antibody in a precipitate becomes important when it is realized that some classes of antibodies, and antibodies from some species, do not precipitate well even in the presence of "optimal" proportions of antigen and antibody. A number of reactions do exist that measure the primary binding between antiserum and low molecular weight haptens employing modifications of equilibrium dialysis techniques.[69] However, the application to large macromolecules has been more difficult to develop. The ammonium sulfate test (Farr) was developed with the antigen bovine serum albumin (BSA), which was labeled with ^{131}I, to fill the need for a primary binding test suitable for nondialyzable large macromolecules.[70] As devised it can measure the capacity of antisera to combine with the soluble macromolecular antigens and can detect both precipitating and nonprecipitating antibody. The principle of the reaction depends upon the fact that the antigen (BSA) is soluble in 50% saturated ammonium sulfate, whereas antigen–antibody complexes, which assume the solubility properties of the antibody, are insoluble under the same conditions.

One of the serious limitations of this technique is the need for the antigen to be soluble in 50% saturated ammonium sulfate. However, modifications have been developed employing anti-immunoglobulin serum (rather than ammonium sulfate), which precipitates the soluble complexes.[71] The anti-immunoglobulin serum is directed against the immunoglobulins of the specific species being tested. This reagent must be checked for its ability to precipitate all the immunoglobulin in the serum being assayed. The precipitation of the radioactive antigen in the presence of increasing dilutions of serum is a measure of the amount of the antibody; that is, the greater the dilution of antiserum against a specific immunogen that still combines with and precipitates a constant amount of antigen, the greater is the strength of the serum. This double-antibody or radioimmunoprecipitation test has been used to measure a variety of hormonal, microbial, and tumor antigens.

Both the ammonium sulfate and anti-immunoglobulin techniques have been used not only to measure the presence or the concentration of antibody, but to detect very small amounts of antigen in fluids or solutions. (Discussions and applications of the various radioimmunoassay procedures referred to here are given elsewhere in this volume.)

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[3] The Experimental Induction of Antibodies to Nucleic Acids

By B. DAVID STOLLAR

Antibodies to nucleic acids have found many uses in the specific measurement of naturally occurring or modified nucleic acids both in solution and *in situ*. To obtain the required antibodies, it has been necessary to link nucleic acids or their components to carrier proteins or synthetic polypeptides to form immunizing complexes because injection of purified nucleic acids alone into normal animals does not stimulate significant antibody production. Once the antibodies are formed, they react with the nucleic acid in the absence of carrier.

A variety of immunogens have been developed, with either small fragments, such as nucleotides or oligonucleotides conjugated covalently to proteins, or with high molecular weight polynucleotides in physical complexes with protein carriers. With these immunogens, antibodies specific for each of the normal bases of DNA and RNA, or for modified bases or base sequences, have become available as selective reagents. Antibodies that recognize helical shapes have also become available. The applications of anti-nucleic acid antibodies have included localization of specific modified bases in ribosomes[1] or chromosomes[2]; identification of denatured DNA in replicating DNA *in situ*[3]; studies of the denaturation and renaturation of DNA[4]; identification of double-stranded RNA intermediates of viral replication[5]; gene localization by *in situ* hybrid detection[6]; isolation of DNA enriched in specific genes[7]; measurement of ultraviolet

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